Total Chemical Synthesis of a Unique Transcription Factor-Related Protein: cMyc−Max

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Received October 28, 1994®

Abstract: cMyc and Max are proteins that regulate gene expression by associating to form noncovalent dimers that bind to specific regions of double-stranded DNA, thus activating or inhibiting mRNA transcription. Each of these transcription factor proteins contains an ~90 amino acid residue basic/helix−loop−helix/zipper (b/HLH/Z) domain responsible for formation of the protein dimers and for DNA binding. Noncovalent heterodimers of the cMyc and Max b/HLH/Z domains are believed to be functionally important *in vivo* but have not been accessible by conventional means (recDNA expression). Here we report the total chemical synthesis of novel covalently-linked dimers of the b/HLH/Z domains of these transcription factors by a modular strategy based on the convergent chemical ligation of several unprotected peptide segments. The resulting ~20 kDa synthetic proteins were purified to homogeneity and characterized by electrospray mass spectrometry. The synthetic covalent cMyc−Max heterodimer and the covalent Max homodimer control were shown to bind DNA specifically at the region involved in the regulation of mRNA transcription. Access to defined heterodimeric forms of the b/HLH/Z transcription factors will allow the structural and functional properties of these important protein regulators of gene expression to be studied.

Introduction

The vertebrate nuclear proteins cMyc and Max are transcription regulation factors. cMyc is implicated in the control of normal cell proliferation and differentiation as well as in neoplastic transformation.[1] Interaction of these proteins with DNA plays a central role in controlling mRNA transcription, and their study is essential to understanding the regulation of gene expression. The cMyc-related transcription factors are characterized by a domain containing the basic/helix−loop−helix/zipper (b/HLH/Z) dimerization and DNA binding motif. With many transcription factors, including the b/HLH/Z family, protein dimerization is a prerequisite for DNA binding.[2,3] The basic region comprises the highly conserved DNA binding domain, while the helix−loop−helix and leucine zipper domains provide dimerization interfaces.

cMyc does not form stable homodimers at physiological protein concentration.[4] Consequently, cMyc alone is not adequate for DNA binding and cell transformation. cMyc does form noncovalent heterodimers with Max. The resulting heterodimer binds double-stranded DNA in a sequence specific manner at the palindromic sequence known as an E-box $\begin{Bmatrix}\text{GTNNAC}\\ \text{CANNTG}\end{Bmatrix}$ and activates transcription.[4] In contrast, Max forms stable homodimers that bind the same DNA sequence but do not activate transcription, thus antagonizing the activity of the cMyc−Max heterodimer by competing for the same target sequence.[1,5] Max also forms heterodimers with other b/HLH/Z proteins, such as Mad[6] and Mxi1,[7] indicating that Max may play a central role in regulating the activity of a network of transcription factors. Homo- and heterodimer formation is entirely determined by the HLH/Z domains of the interacting proteins, creating a variety of protein complexes capable of binding DNA, but with different consequences.[2] The ability to control which dimers are formed provides a mechanism for the regulation of cellular function and development.

The structure of the b/HLH/Z domain of the noncovalent, bacterial Max homodimer complexed with its target DNA sequence has been determined by X-ray crystallography to 2.9 Å resolution.[8] This structure provides an important foundation on which to build a better understanding of DNA recognition and dimerization of b/HLH/Z transcription factors. The obvious next step is the study of the functionally-relevant heterodimer forms of these transcription factors.[9,10] However, to date, biophysical studies of b/HLH/Z heterodimers have been complicated by unwanted homodimer formation at supraphysiologic protein concentration, effectively preventing access to these systems. Total chemical synthesis of covalently-linked heterodimers on a sufficiently large scale represents a means of overcoming this fundamental limitation. Moreover, this approach will readily permit preparation of point mutants, inclusion of unnatural amino acids, and a variety of other structural modifications. The synthetic route will also avoid problems of protein stability and toxicity sometimes found with *E. coli* expression systems.

Important advances in peptide synthesis over the past several years have made total chemical synthesis a feasible route to the construction of native proteins and proteins of extraordinary

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® Abstract published in *Advance ACS Abstracts*, February 15, 1995.

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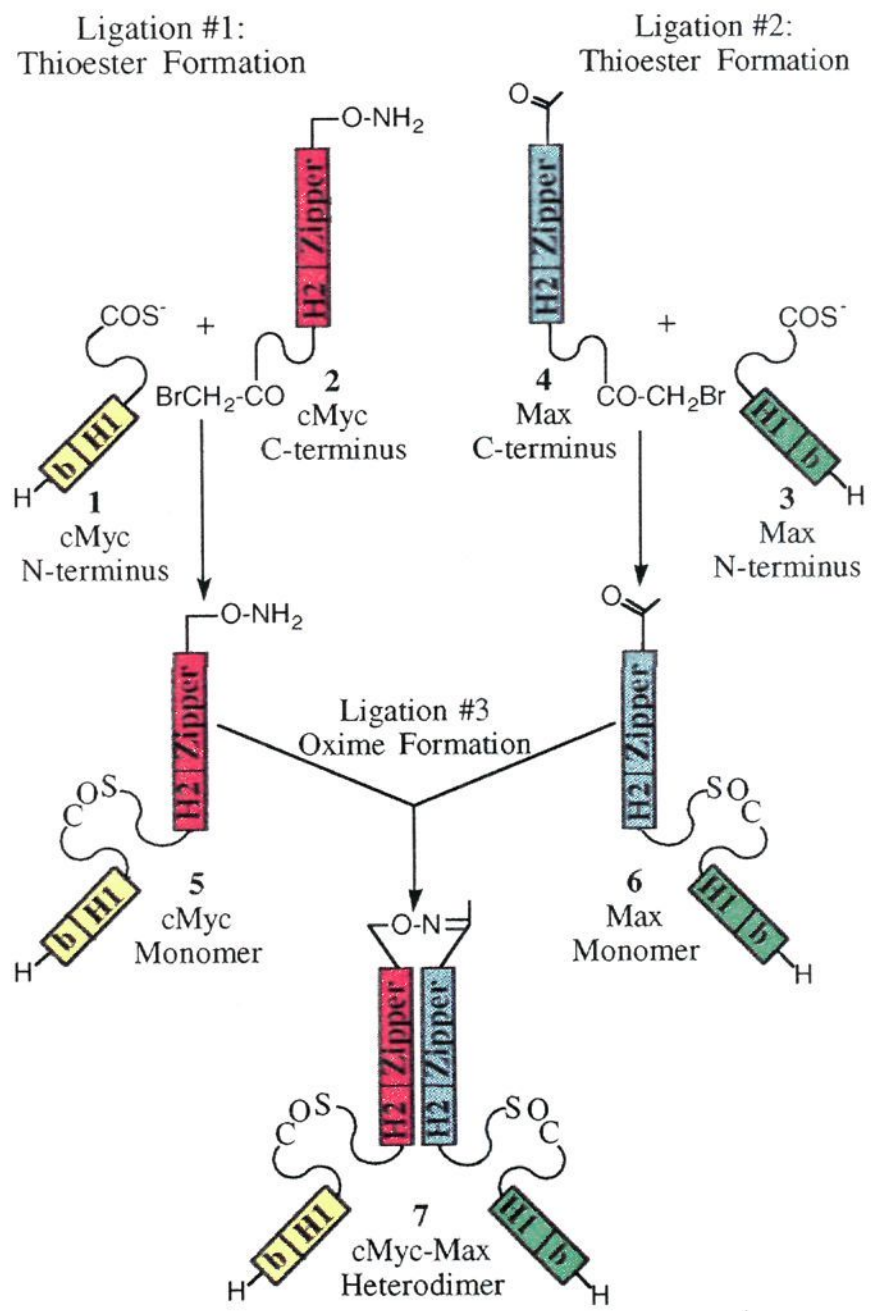
structure.[11] Recently, we introduced the chemical ligation approach to the total chemical synthesis of proteins.[12,13] This method involves the joining of unprotected peptide segments in an unambiguous manner by the chemoselective reaction of unique, mutually reactive functionalities, one on each segment. In this way a stable bond is formed in a predictable and controlled fashion even in the presence of the range of functional groups usually found in peptides. Chemical ligation is not limited to formation of a peptide bond at the ligation site,[13] a variety of ligation chemistries can be used to implement the strategy. Thus, thioester-forming ligation reactions have been used in the synthesis of fully functional HIV-1 protease,[12] as well as related analogue molecules for the study of enzyme mechanisms,[14] and in the preparation of a four-helix template-assembled synthetic protein molecule (TASP).[15] Oxime-forming ligations have been employed in the production of peptide dendrimers.[16] Other chemistries of potential utility in the chemical ligation approach include hydrazone formation,[17] metal chelation,[18] disulfide formation,[19,20] thioether formation,[21] and thiazolidine ring formation.[22] The synthesis of functional protein analogues containing unnatural backbone elements represents an important conceptual breakthrough that demonstrates that we need not be restricted to the formation of native peptide bonds in order to have a biologically active protein.

Syntheses of even larger and more complex protein analogues would be possible through an approach involving the use of two mutually compatible ligation chemistries. This would allow the condensation of three or more unprotected peptide segments in a specific manner, according to the well-known principle of convergent organic synthesis. The thioester-[12] and oxime-forming[16] chemistries are in principle two such mutually compatible ligation methods. In the work described in this paper, we have used these ligation techniques in a modular approach to the synthesis of truncated, covalently-linked transcription factor dimers. This has been accomplished for the b/HLH/Z domains of the cMyc−Max heterodimer and the Max homodimer to yield biochemically active protein molecules that bind the expected DNA target sequence.

Results and Discussion

Design. The target dimeric protein molecules contain 172 amino acid residues and are made up of truncated forms of the ~90 residue b/HLH/Z domains of cMyc or Max covalently joined to form a single protein-like entity with two amino terminals. The monomer amino acid sequences are shown in Figure 1. The strategies for the total chemical syntheses of the covalently-linked hetero- and homodimers are outlined in Schemes 1 and 2, respectively. In both cases, the resulting

Scheme 1. Synthesis of Covalently-Linked cMyc−Max Heterodimer by Convergent Chemical Ligation



dimers contain three ligation sites: a thioester linkage *within* each monomer and an oxime linkage *between* the two monomer polypeptide chains. Figure 2 shows a model of the covalently-linked Max homodimer highlighting the location of the ligation sites.[8] This particular combination of ligations was accomplished through the use of four functional groups: thio acid and bromoacetyl groups that react to form the thioester linkages,[12] and (aminooxy)acetyl and ketone functionalities that react to form the oxime linkage.[16] The intradomain thioester ligation sites were located in the loop region of the b/HLH/Z domain. This region does not contribute significantly to DNA binding or to dimerization and is known to tolerate variations in size and sequence.[8,23] We made use of this tolerance to replace amino acids in this loop region at the thioester ligation site, for reasons of synthetic convenience.

A ketone-containing side chain acyl moiety was used as one component in the oxime-forming ligation. This avoided the use of periodate oxidation of the peptide,[16] with the possibility of attendant undesired oxidative side reactions (e.g., at Met residues). Model studies showed that the product ketoxime was stable between pH 4.5 and pH 8. The oxime linkage was used to tether the carboxyl-terminal regions of the monomer polypeptide chains to yield a protein analogue with two free amino terminals. The carboxyl-terminal regions of the b/HLH/Z domains are disordered in the Max cocrystal structure,[8] and these regions were therefore thought likely to tolerate the presence of an unnatural tether.

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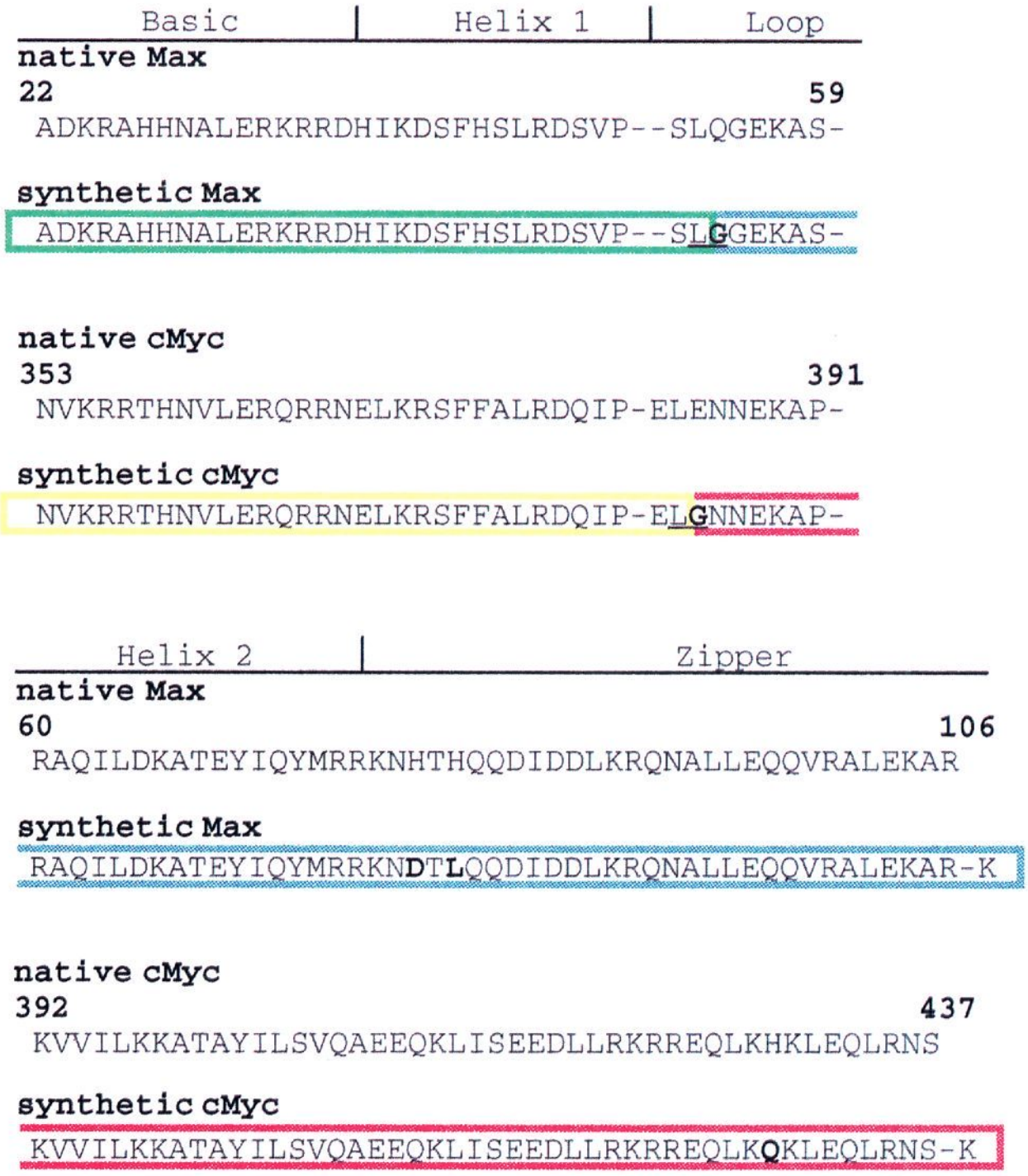

Figure 1. Native and synthetic target amino acid sequences of Max[33] and cMyc.[32] The thioester ligation sites in the synthetic proteins are underlined. Amino acid replacements in the synthetic proteins are shown in bold.

In the case of the heterodimer (Scheme 1), the thioester reactions were performed first, and the resulting monomers (**5** and **6**) were purified and then used in the oxime ligation to produce the desired covalent heterodimer **7**. In contrast, for the homodimer **9** (Scheme 2), since the amino terminal segment **3** of both monomers is the same, there was no need for separate thioester ligations, and it was possible to use a self-assembly "autosplicing" approach in which all three peptide segments (**3**, **4**, and **8**) were combined in one reaction mixture. The thioester and oxime ligations occurred simultaneously to produce the desired homodimer **9**.

Synthesis of Unprotected Peptide Segments. All peptide segments were synthesized by stepwise solid-phase methods using machine-assisted *in situ* neutralization/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation protocols for *tert*-butoxycarbonyl (Boc) chemistry according to published procedures.[24] The synthesis of the peptide segments **1** and **3** is outlined in Scheme 3. The thioester resin necessary for the generation of a peptide-$^{\alpha}$COSH was prepared by a modification of the method of Yamashiro.[25] The thiol **11** was synthesized from the reaction of the chloride **10** with thiourea followed by hydrolysis of the resulting thiouronium salt in aqueous NaOH. Thioester **12** resulted from the reaction of **11** with the succinimide ester of Boc-L-leucine. Thioester **12** was then coupled to aminomethyl-resin with HBTU to give leucine thioester resin **13**. Assembly of the rest of the peptide chain was carried out in stepwise fashion to give the peptide-resin **14**. Acidolytic deprotection and cleavage of **14** in anhydrous HF gave the unprotected thioacid peptides **1**(cMyc) and **3**(Max), each of which was 32 amino acids in length.

With procedures for the synthesis of these peptide-$^{\alpha}$COSH species well established in our laboratory,[12,14,15,26] the key to the synthesis of the covalently-linked dimers became the synthesis of the peptide segments **2, 4,** and **8** containing functional groups at *both* the amino and carboxyl terminals. The strategy used in the synthesis of these bifunctional peptide segments is outlined in Scheme 4. The (aminooxy)acetyl- or ketone-containing functionalities were coupled to the peptide at the side chain amine of a carboxyl-terminal lysine residue, while the bromoacetyl groups were coupled to the α-NH_2 group of each peptide. This was accomplished through the use of the orthogonal amine protecting groups, 9-fluorenylmethyloxycarbonyl (Fmoc, base labile) and Boc (acid labile). Boc-Lys(Fmoc)-OH was coupled to 4-methylbenzhydrylamine (MBHA) resin to produce the protected lysine resin **15**. Stepwise chain

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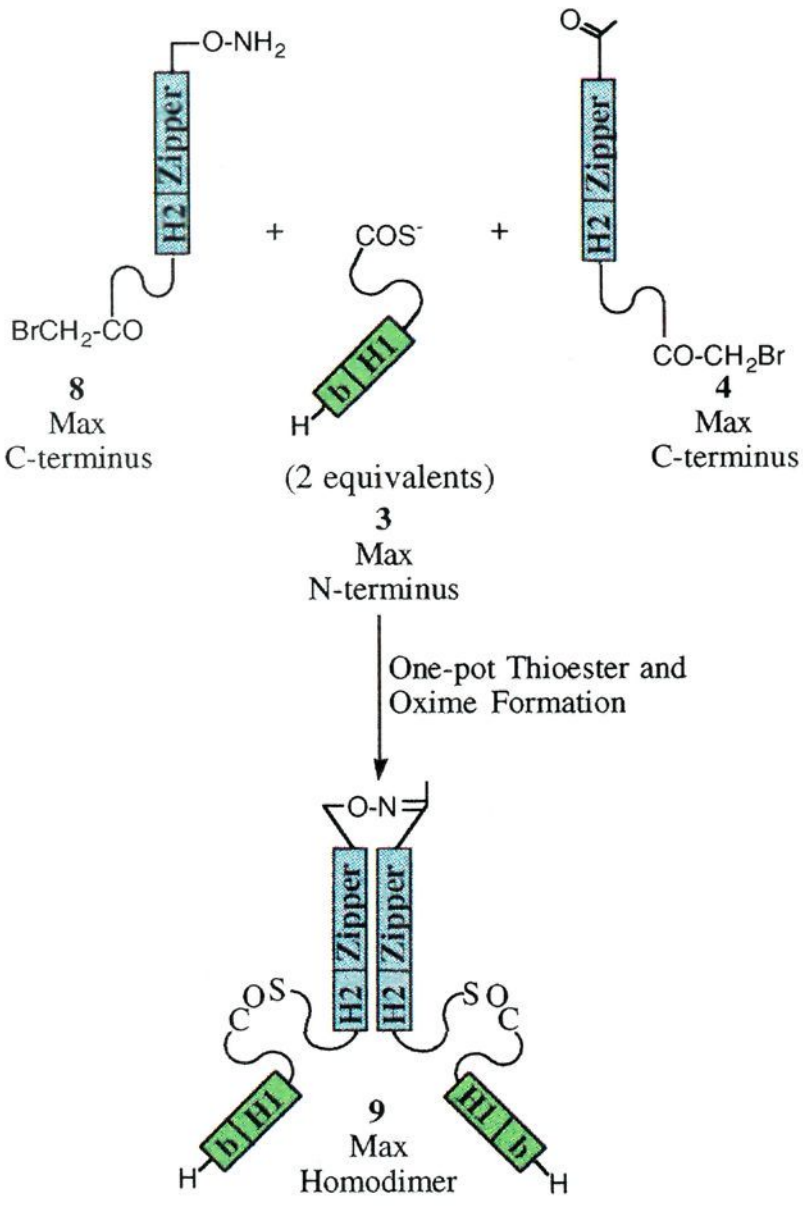
Scheme 2. Synthesis of Covalently-Linked Max Homodimer by "Autosplicing" Chemical Ligations



assembly was performed at this stage. Once chain assembly was complete, the Fmoc group on the side chain amine of the carboxyl-terminal lysine was removed with 20% piperidine in *N*,*N*-dimethylformamide (DMF) to produce the peptide-resin **16**.

For the (aminooxy)acetyl peptides, the succinimide ester of 2-chlorobenzyloxycarbonyl (2-Cl-Z)-protected (aminooxy)acetic acid (**17**) was coupled to **16** to give the peptide-resin **19**. Surprisingly, the 2-Cl-Z-protected (aminooxy)acetic acid derivative was found to be labile to base treatment and thus not stable to the coupling conditions (*in situ* neutralization/HBTU activation) used in stepwise assembly of the peptide chain.[24] Accordingly, it was essential that the (aminooxy)acetic acid be coupled to the peptide *after* chain assembly was complete. The amino-terminal Boc group was then removed from **19** with trifluoroacetic acid, (TFA) and the resulting free amine reacted with the symmetric anhydride **21** produced from the reaction of bromoacetic acid and 1,3-diisopropylcarbodiimide (DIC).[27] Because the bromoacetyl group will react with piperidine, removal of the Fmoc group and the subsequent reaction of the resulting free amine with the appropriate compound (**17** or **18**) must be performed *prior* to bromoacetylation. Finally, the bromoacetylated peptide-resin was deprotected and cleaved from the resin in anhydrous HF to give the segments **2**(cMyc) or **8**(Max).

For the peptides with carboxyl-terminal ketones, peptide-resin **16** was reacted with the symmetric anhydride (**18**) produced from the reaction of 4-oxopentanoic acid with DIC. The rest of the synthesis was identical to that of the (aminooxy)acetyl peptides with deprotection and cleavage from the resin giving

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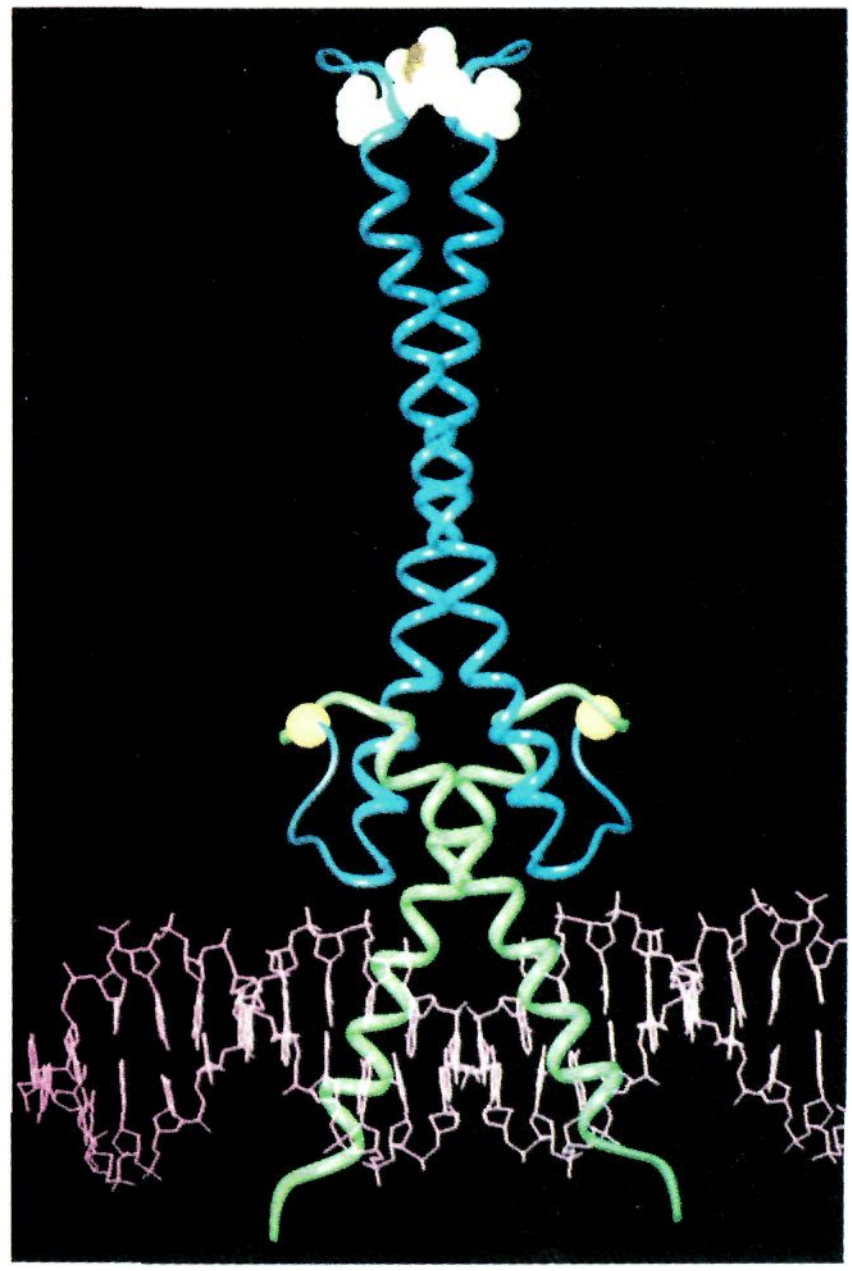

Figure 2. Model of the covalently-linked Max homodimer based on the X-ray crystal structure of bacterial Max.[8] The figure was created using a combination of the molecular modeling programs *Insight II* (Biosym) and *Sculpt* (created by Dr. Mark Surles of Interactive Simulations, Inc.). The color coding is consistent with Scheme 2, with the carboxyl-terminal segments shown in blue and the amino-terminal segments shown in green. The thioester ligation sites are shown in yellow. The oxime-containing linker at the carboxyl termini, consisting of covalently-linked, side-chain-modified lysine residues, is shown in white with the three atoms of the oxime (O−N=C) shown in red. Duplex DNA is shown in purple.

the bromoacetylated peptide **4**(Max). The peptide segments **2**, **4**, and **8** corresponding to the carboxyl-terminal region of the monomer b/HLH/Z domains were each 53 amino acids in length.

The preceding methods were compatible with all the amino acid protected side chains used in the synthesis of peptide segments **2** and **8**, with the exception of histidine. Dinitrophenyl (DNP)[28,29] and benzyloxymethyl (Bom)[30] have been found to be useful protecting groups for the protection of the imidazole (Im) side chain of Boc-His for use in solid phase peptide synthesis (SPPS). The Im DNP group is stable to HF and is usually removed by thiolysis prior to cleavage of the peptide from the resin. Unexpectedly, the 2-Cl-Z-protected (aminooxy)-acetyl functionality in resin-bound segments **2** and **8** was sensitive to nucleophilic attack, the 2-Cl-Z group being removed by the mercaptoethanol used to remove the Im DNP group. On the other hand, the Bom group is cleaved in HF, but formaldehyde is generated in the process. This formaldehyde reacted with the deprotected (aminooxy)acetyl group, rendering it unavailable for reaction with the 4-oxopentanoyl peptide (**4**).

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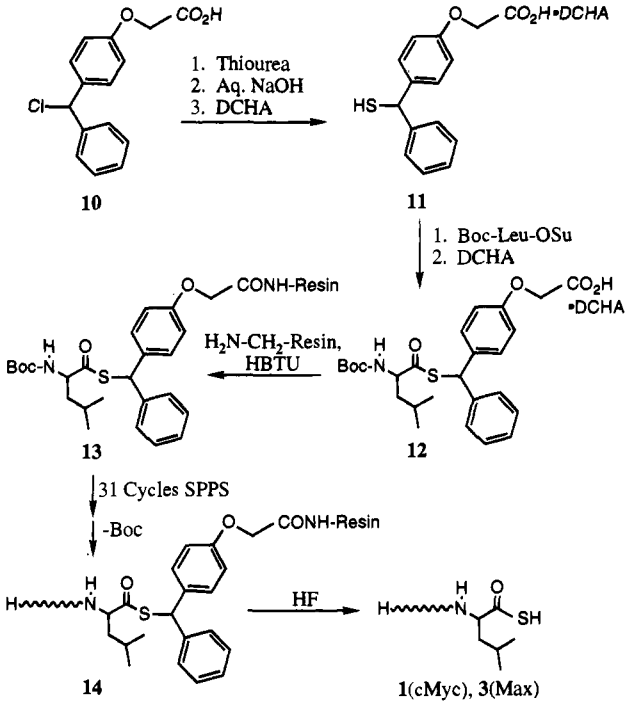
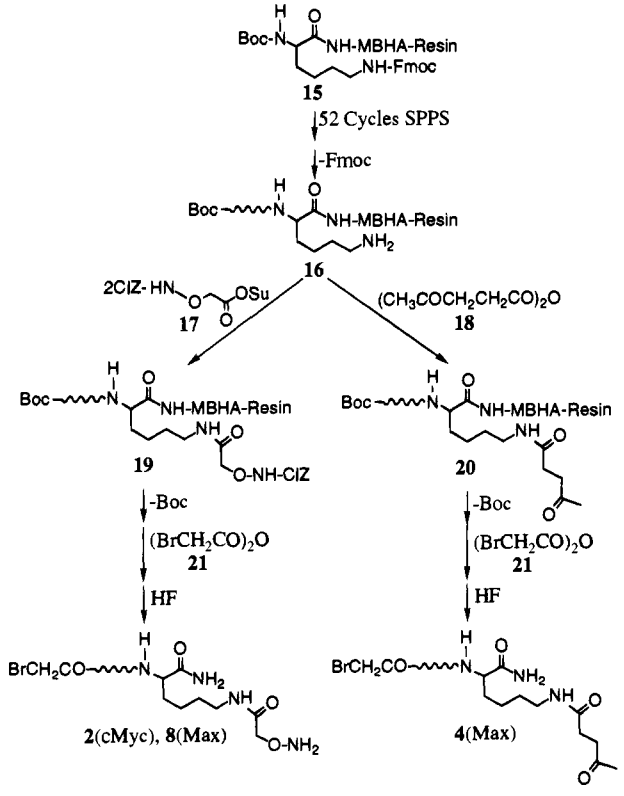
Scheme 3. Synthesis of Peptide Segments from the Amino-Terminal Region of the b/HLH/Z Domains



Scheme 4. Synthesis of Peptide Segments from the Carboxyl-Terminal Region of the b/HLH/Z Domains



In the syntheses described, these problems were avoided by replacing the His residues in segments **2** and **8** with other amino acids. There are two His residues in the native amino acid sequence of the carboxyl terminal region of the b/HLH/Z domain of Max and one His residue in the corresponding segment of cMyc (Figure 1). In Max, His^{81} is in the position corresponding to the first Leu residue of the leucine zipper region, so a Leu residue was substituted. For the His^{79} in Max, we chose an Asp from the amino acids found in other variants.[8] For synthetic convenience, the His residues in the Max-derived segment **4** were also replaced. For His^{429} in cMyc, the corresponding residue (Gln) in the aligned sequence of Max (Figure 1) was used. All of these residues were in nonconserved positions, and their replacement in the segments **2**, **4**, and **8** had no obvious effect on the ability of the covalently-linked dimers to bind DNA. As an additional note, the (aminooxy)acetyl group will react with common laboratory aldehydes and ketones, such as acetone, to form stable oximes. Therefore, much care must be taken to avoid accidental exposure of the (aminooxy)acetyl peptides to these compounds.

Synthesis of the cMyc and Max Monomers (5 and 6): Thioester-Forming Ligations. In the ligation reactions giving the cMyc (**5**) and Max (**6**) monomers, the peptide segments **1** and **3** contained a carboxyl-terminal -Leu-$^{\alpha}$COSH and were used in excess. The resulting ligations between these peptides and the amino-terminal bromoacetyl groups of the segments **2** and **4** gave a -Leu[COS]Gly- sequence at the site of ligation (Figure 1). Note that the Gly residue at the ligation site (replacing residue Gln^{54} in Max and replacing residue Glu^{385} in cMyc) derives from the bromoacetyl moiety. Thus, condensation of a 32-residue -$^{\alpha}$COSH segment with a 53-residue bromoacetylated segment gave an 86-residue product. All ligations were performed in acidic (pH 4.7) aqueous buffer. The time course of the ligation forming the cMyc monomer (**5**), as monitored by analytical reverse-phase HPLC, is shown in Figure 3A. The ligation was rapid (nearly complete within 1 h), and clean, with little or no generation of undesirable side products. Likewise, the ligation reaction to form the Max monomer (**6**), as shown in Figure 4A, was nearly complete within 1 h. Each monomer was readily purified by semipreparative reverse-phase HPLC and lyophilized to give a white solid. Final products were characterized by electrospray MS (Figures 3B and 4B). The cMyc monomer (**5**) gave a mass of 10 396 ± 2 Da [calcd 10 396 Da (average isotope composition)], while the Max monomer (**6**) gave a mass of 10 233 ± 2 Da [calcd 10 236 Da (average isotope composition)].

Synthesis of the cMyc–Max Heterodimer (7): Oxime-Forming Ligation. The ligation to give the oxime-linked cMyc–Max heterodimer was performed by reacting the purified monomers **5** and **6** in acidic (pH 4.7) aqueous buffer. The course of the reaction, as followed by analytical reverse-phase HPLC, is shown in Figure 5A. Like the thioester-forming ligations, the reaction was clean with little or no side product formation, and the resulting heterodimer was readily purified by semipreparative reverse-phase HPLC and lyophilized to give a white solid. However, compared to the thioester-forming ligations, the oxime formation was slower and incomplete. The reaction slowed to an insignificant rate after 24 h, and product (**7**) was obtained in 21% yield after purification. The final product (**7**) was characterized by electrospray MS (Figure 5B) to give a mass of 20 610 ± 3 Da [calcd 20 614 Da (average isotope composition)].

Synthesis of the Max Homodimer (9): "Autosplicing" Ligation. The reaction to form the Max homodimer was carried out in acidic (pH 4.7) aqueous buffer. All three peptide segments (**3**, **4**, and **8**) were added simultaneously with the segment **3** in excess. The course of the reaction is shown in Figure 6A. After 3 h, the desired homodimer (peak **9**) and the two monomers, (aminooxy)acetylated Max monomer and 4-oxopentanoylated Max monomer (peaks **a** and **6**, respectively), products of the thioester-forming ligations, were identified by electrospray MS. However, neither of the bromoacetyl segments (**4** and **8**) were detected, indicating that these compounds had

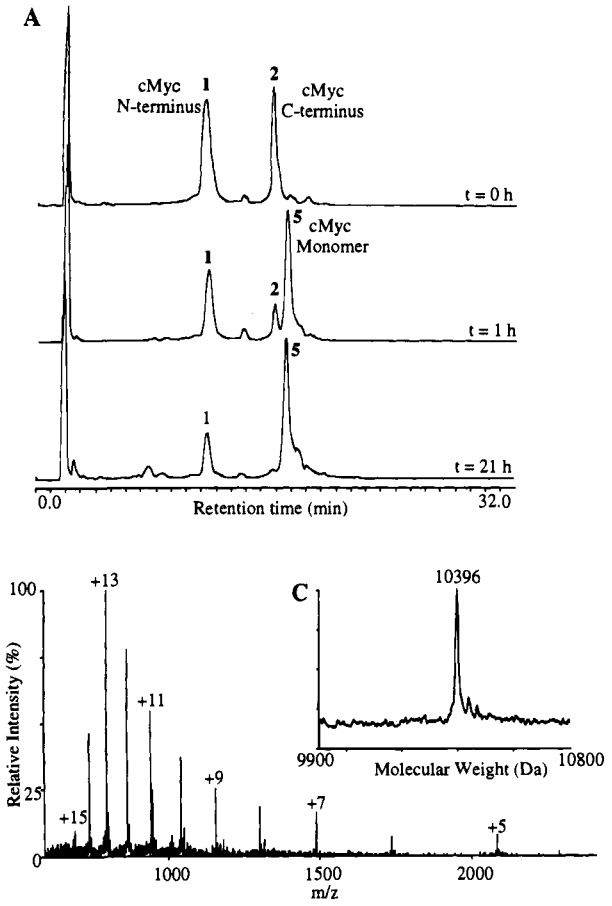


Figure 3. Synthesis of the cMyc monomer by chemical ligation in 8 M urea and 0.1 M Na_2HPO_4 (pH 4.7). (A) Analytical HPLC at $t = 0$, 1, and 21 h of reaction mixture kept at 4 °C. Peaks are labeled with numbers consistent with the peptide segments shown in Scheme 1: **1**, cMyc(353–384)-$^{\alpha}COS^{-}$; **2**, $BrCH_2CO$-cMyc(386–437)-Lys-$^{\epsilon}N(COCH_2ONH_2)$; **5**, ligation product, cMyc(353–437)-Lys-$^{\epsilon}N(COCH_2ONH_2)$. (B) Electrospray MS of HPLC-purified peak **5** (cMyc monomer) showing the distribution of protonation states of the molecule. (C) Hypermass reconstruction of the raw MS data to a single charge state; the cMyc monomer ligation product had an observed mass of 10 396 ± 2 Da [calcd 10 396 Da (average isotope composition)].

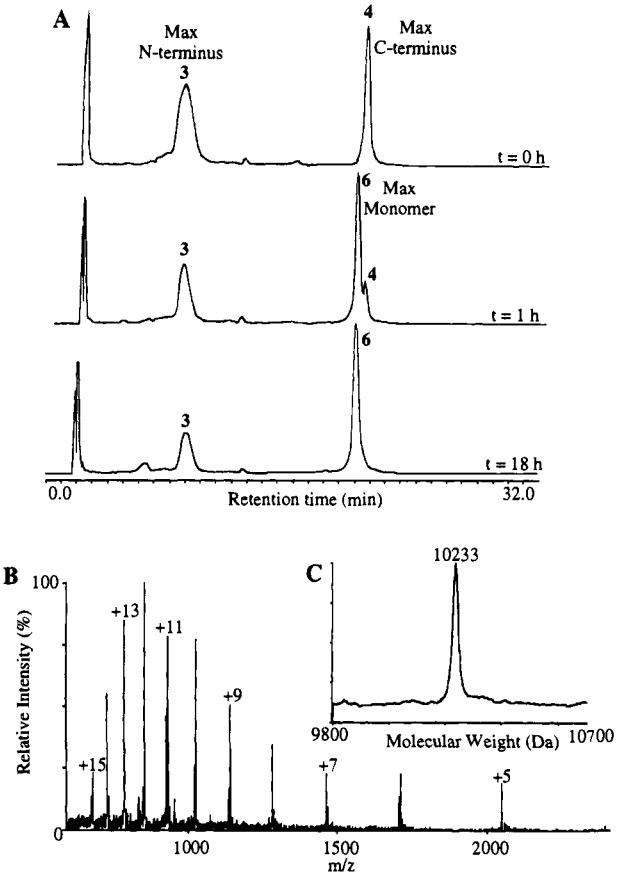


Figure 4. Synthesis of the Max monomer by chemical ligation in 8 M urea and 0.1 M Na_2HPO_4 (pH 4.7). (A) Analytical HPLC at $t = 0$, 1, and 18 h of reaction mixture kept at 4 °C. Peaks are labeled with numbers consistent with the peptide segments shown in Scheme 1; **3**, Max(22–53)-$^{\alpha}COS^{-}$; **4**, $BrCH_2CO$-Max(55–106)-Lys-$^{\epsilon}N(COCH_2CH_2COCH_3)$; **6**, ligation product, Max(22–106)-Lys-$^{\epsilon}N(COCH_2CH_2COCH_3)$. The broad peak observed for segment **3** reflects its behavior under these HPLC conditions; by ESMS, **3** was homogeneous and of high purity. (B) Electrospray MS of HPLC-purified peak **6** (Max monomer) showing the distribution of protonation states of the molecule. (C) Hypermass reconstruction of the raw MS data to a single charge state; the Max monomer ligation product had an observed mass of 10 233 ± 2 Da [calcd 10 236 Da (average isotope composition)].

completely reacted. This is consistent with what was observed in the set of consecutive, separate ligation reactions used to make the cMyc–Max heterodimer, where the thioester-forming ligations proceeded at a faster rate than the oxime formation. After 26 h, (aminooxy)acetylated Max monomer (peak **a**) was nearly depleted and there was no significant formation of side products, definitively illustrating that the thioester and oxime chemistries are mutually compatible. The product homodimer (**9**) was obtained in 20% yield after purification by semipreparative reverse-phase HPLC. The final product (**9**) was characterized by electrospray MS (Figure 6B) to give a mass of 20 425 ± 3 Da [calcd 20 428 Da (average isotope composition)].

Biochemical Activity: Electrophoretic Mobility "Gel Shift" Assay. The covalently-linked cMyc–Max heterodimer (**7**) and Max homodimer (**9**) were examined for their ability to bind DNA at an E-box sequence (radiolabeled duplex DNA of the sequence ctactagCACGTGctagtag) in electrophoretic mobility gel shift assays (Figure 7). Experiments were performed without inhibitor, in the presence of specific inhibitor (unlabeled duplex E-box sequence), or in the presence of nonspecific double-stranded DNA (duplex of the sequence gaaaatcacccaactgca). The DNA binding profiles of the synthetic covalent dimers were compared to that of the noncovalent bacterial Max homodimer (Figure 7). Both the synthetic covalent cMyc–Max heterodimer–DNA (lane 8) and Max homodimer–DNA (lane 5) complexes migrate to positions similar to those in the bacterial Max–DNA complex (lane 2) and display the same specificity regarding competition as bacterial Max (lanes 6, 7, 9, and 10 vs lanes 3 and 4).

Significance. The novel cMyc- and Max-derived synthetic protein constructs displayed specific DNA binding properties, despite containing a variety of amino acid replacements, novel backbone structures, and an unnatural topology. The explanation for this lies in the domain structure of proteins. Native proteins often consist of autonomous structural/functional units (frequently referred to as "domains").[31] In many cases, the identity of the residues that connect these domains seems to not be critical, and therefore, these connecting loops can be replaced by a variety of structures, natural or unnatural, as long as the independent functional units remain in the correct orientation relative to each other. With the b/HLH/Z transcription factors studied here, the basic region and the first helix were viewed as one functional unit (DNA binding) and the second helix and the leucine zipper were viewed as a second functional unit (dimerization). Sequence variability was ob-

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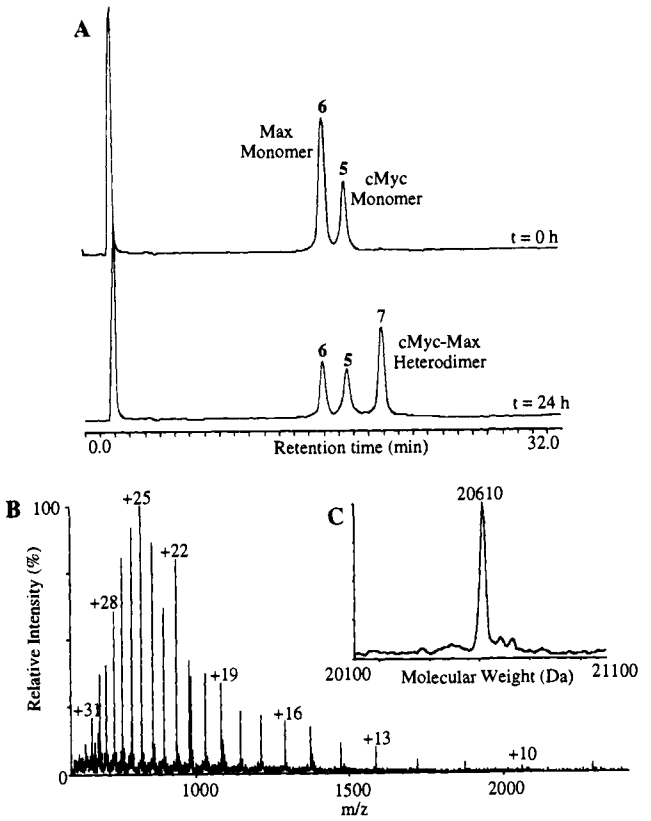

Figure 5. Synthesis of the cMyc−Max covalent heterodimer by chemical ligation in 8 M urea and 0.1 M Na_2HPO_4 (pH 4.7). (A) Analytical HPLC at t = 0 and 24 h of the reaction mixture kept at room temperature. Peaks are labeled with numbers consistent with the peptide segments shown in Scheme 1: **5**, monomeric [$(COS)^{384-385}$] cMyc(353−437)-Lys-$^{\epsilon}$N($COCH_2ONH_2$), **6**, monomeric [$(COS)^{53-54}$] Max(22−106)-Lys-$^{\epsilon}$N($COCH_2CH_2COCH_3$); **7**, ligation product, covalently-linked cMyc−Max heterodimer. (B) Electrospray MS of HPLC-purified peak **7** (cMyc−Max covalent heterodimer) showing the distribution of protonation states of the molecule. (C) Hypermass reconstruction of the raw MS data to a single charge state; the cMyc−Max covalent heterodimer ligation product had an observed mass of 20,610 ± 3 Da [calcd 20 614 Da (average isotope composition)].

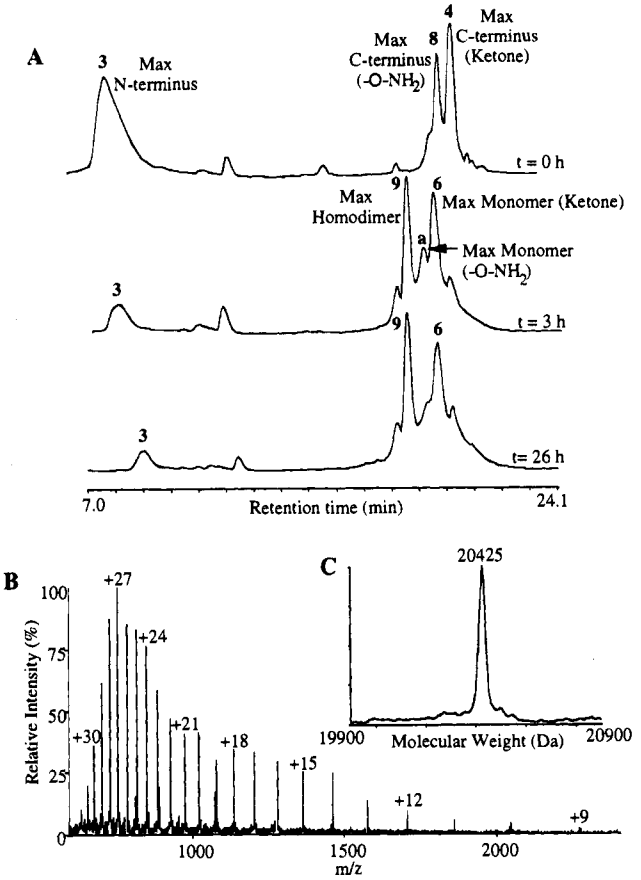

Figure 6. Synthesis of the Max covalent homodimer by "autospliting" chemical ligation in 8 M urea and 0.1 M Na_2HPO_4 (pH 4.7). (A) Analytical HPLC at t = 0, 3, and 26 h of the reaction mixture kept at room temperature. Peaks are labeled with numbers consistent with the peptide segments shown in Schemes 1 and 2: **3**, Max(22−53)-$^{\alpha}COS^{-}$; **4**, $BrCH_2CO$-Max(55−106)-Lys-$^{\epsilon}$N($COCH_2CH_2COCH_3$); **8**, $BrCH_2CO$-Max(55−106)-Lys-$^{\epsilon}$N($COCH_2ONH_2$); **6**, intermediate ligation product, monomeric [$(COS)^{53-54}$] Max(22−106)-Lys-$^{\epsilon}$N($COCH_2CH_2COCH_3$); **9**, final ligation product, covalently-linked Max homodimer. The additional peak **a** is an intermediate ligation product, monomeric [$(COS)^{53-54}$] Max(22−106)-Lys-$^{\epsilon}$N($COCH_2ONH_2$) [observed mass 10 208 ± 2Da, calcd 10 210 Da (average isotope composition)]. (B) Electrospray MS of HPLC-purified peak **9** (Max covalent homodimer) showing the distribution of protonation states of the molecule. (C) Hypermass reconstruction of the raw MS data to a single charge state; the Max covalent homodimer ligation product had an observed mass of 20 425 ± 3 Da [calcd 20 428 Da (average isotope composition)].

served in the connecting loop region, making this an ideal ligation site within each monomer. As for the ligation joining the two monomers at their carboxyl-terminal ends, since there is no native connecting polypeptide chain to replace, a great deal of structural variability could probably have been tolerated in that area, as illustrated by the normal biochemical function of the oxime-linked dimer constructs. The fact that these highly modified transcription factor synthetic constructs show the same DNA binding properties as the noncovalent homodimer bacterial protein demonstrates that we need not be restricted to native peptide bonds in order to have a biologically active protein, as long as appropriate sites are chosen as ligation points.

This study demonstrates that a modular approach, wherein independent functional or structural units are joined by chemical ligations, is a feasible method of protein synthesis. This technique can construct proteins of unusual structure with full biological activity, thus providing a powerful new way to study important biological phenomena. The chemical ligation approach to the total chemical synthesis of proteins has the potential to take protein synthesis far beyond the realm of nature. Mutually compatible ligation chemistries, such as the thioester- and oxime-forming reactions reported on here, can be used for the ligation of a number of different unprotected peptide segments in a convergent manner. These techniques allow for the synthesis of larger proteins than were previously possible by chemical means, as well as placing proteins of unnatural structure and topology within our grasp.

The covalently-linked dimers of this study provide an excellent example of the versatility of the chemical synthesis approach to the construction of proteins displaying a topological situation not attainable by linear ribosomal protein synthesis, namely a synthetic protein molecule containing *two* amino-terminal domains. In the case of the b/HLH/Z family of transcription factors, synthetic access to defined covalent heterodimers with natural biochemical activity will allow detailed investigation of the structural biology of this important, but previously inaccessible, class of transcription factors.

Experimental Section

Materials and Methods. Machine-assisted solid-phase peptide syntheses were carried out on a custom-modified Applied Biosystems 430A peptide synthesizer. Reverse-phase HPLC was performed on a Rainin HPLC system with 214 nm UV detection, using Vydac C-18 analytical (5 μm, 0.46 × 15 cm) and semipreparative (10 μM, 1.0 ×

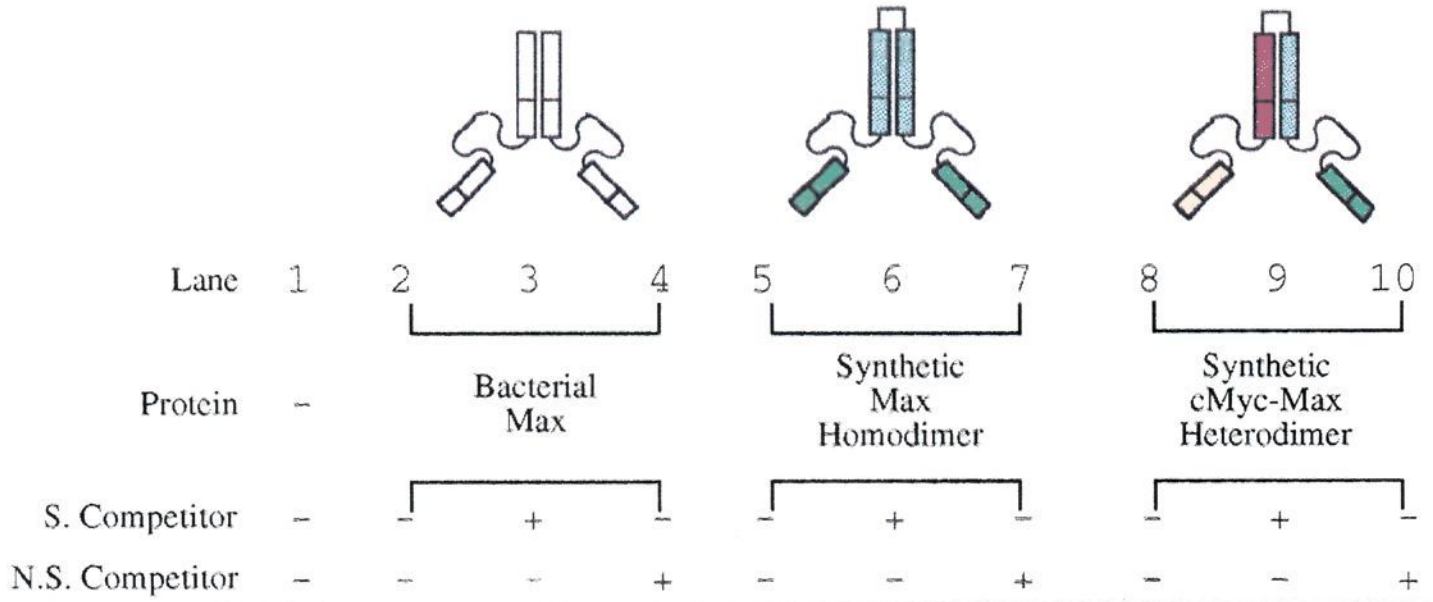

Figure 7. Autoradiogram of electrophoretic mobility "gel shift" assay of the DNA binding activity of the synthetic covalently-linked cMyc−Max and Max−Max dimers. Both synthetic covalent b/HLH/Z dimer protein constructs showed specific DNA binding activity similar to that of the bacterial Max noncovalent dimer, as discussed in the text. Equimolar amounts of annealed radiolabeled double-stranded DNA probe and protein were incubated for 1 h at room temperature before being resolved in a prerun 0.5x TBE 6% acrylamide 29:1 acrylamide: bis(acrylamide) gel at 15 V/cm for 3 h. Bands were visualized by autoradiography. The probe and the unlabeled specific competitor (S) are a 20 bp duplex DNA of the sequence ctactagCACGTGctagtag. The unlabeled nonspecific competitor (N.S.) is a duplex with the unrelated sequence gaaaatcacccaactgca. Competitor DNA was present in 100-fold excess over probe DNA.

25 cm) columns. Preparative reverse-phase HPLC was performed on a Waters Delta Prep 4000 System using a Vydac C-18 column (15−20 μm, 5.0 × 25 cm). Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% TFA in water, B = 90% CH_3CN/10% water containing 0.09% TFA) over 30−60 min at 1 mL/min (analytical), 3 mL/min (semipreparative), or 30 mL/min (preparative). Mass spectra of all peptide segments and proteins were obtained with a Sciex API-III electrospray quadrupole mass spectrometer. Observed masses were derived from the experimental *m/z* values for all observed protonation states of a molecular species, using the program MacSpec (Sciex). Calculated masses were based on average isotope composition and were derived using the program MacProMass (Terry Lee and Sunil Vemuri, Beckman Research Institute, Duarte, CA). All other mass spectrometry was performed at The Scripps Research Institute Mass Spectrometry Facility. ^{1}H NMR spectra were recorded on a Bruker 250 MHz spectrophotometer, and chemical shifts are reported in parts per million downfield from Me_4Si. Microanalyses were performed at The Scripps Research Institute X-ray Crystallographic Facility and agreed with calculated values to within ±0.4%. Boc-L-amino acids and HBTU were purchased from Novabiochem (La Jolla, CA). Aminomethylcopoly(styrene−divinylbenzene) resin and diisopropylethylamine (DIEA) were obtained from Applied Biosystems (Foster City, CA), and MBHA resin was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). Synthesis-grade DMF was obtained from Mallinckrodt and AR-grade CH_2Cl_2 obtained from Fisher. TFA was obtained from Halocarbon (New Jersey). HF was purchased from Matheson Gas. HPLC-grade CH_3CN was obtained from EM Science or from Fisher. Flash chromatography was performed with silica gel 60 (230−400 mesh) obtained from EM Science. (Aminooxy)-acetic acid hemihydrochloride was purchased from Fluka. All other reagents were AR grade or better and were obtained from Aldrich Chemical or from Fisher.

Peptide Segment Synthesis. Peptides were synthesized in stepwise fashion by established machine-assisted solid-phase methods using *in situ* neutralization/HBTU activation protocols for Boc chemistry.[24] Side chain protection was as follows: Boc-Arg(*p*-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(*O*-cyclohexyl)-OH, Boc-Glu(*O*-cyclohexyl)-OH, Boc-His(Bom)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Boc-Gln-OH and Boc-Met-OH were used without side chain protection. Coupling yields were determined by quantitative ninhydrin assay on peptide-resin samples taken under machine control during the chain assembly.[24]

Peptide Segments 1 and 3. Peptide segments corresponding to the N-terminal region of the target molecule were synthesized on Leu-S-resin (**13**), made by coupling **12** (2.0 equiv) and aminomethyl-resin (1 equiv, washed with 10% DIEA in DMF), with HBTU (1.6 equiv) added as an activating agent and DIEA (1 equiv), in DMF for 2−3 h. After chain assembly was complete, peptides were deprotected and simultaneously cleaved from the resin by treatment with HF containing ~5% *p*-cresol and ~5% resorcinol for 1 h at 0 °C to give the peptide-$^{\alpha}$-

COSH. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous Et_2O, dissolved in 50% aqueous AcOH, diluted with water, and purified either immediately or after prior lyophilization.

cMyc(353−384)-$^{\alpha}$COSH (1). The N-terminal cMyc segment, corresponding to residues 353−384 of the complete human cMyc protein[32] (NVKRRTHNVLERQRRNELKRSFFALRDQIPEL), was synthesized on a 0.22 mmol scale to give 0.88 g of peptide-resin (78% of theoretical yield after ninhydrin sampling). Cleavage of the peptide-resin (169 mg, 26.2 μmol) gave, after purification by preparative reverse-phase HPLC (25−50% B over 60 min at 30 mL/min), 23.0 mg (5.7 μmol) of **1** (22% theoretical yield), which was characterized by electrospray MS to give a mass of 4,037 ± 1 Da [calcd 4035 Da (monoisotopic), 4037 Da (average isotope composition)].

Max(22−53)-$^{\alpha}$COSH (3). The N-terminal Max segment, corresponding to residues 22−53 of the complete murine Max protein[33] (ADKRAHHNALERKRRDHIKDSFHSLRDSVPSL), was synthesized on a 0.23 mmol scale to give 1.00 g of peptide-resin (73% of theoretical yield after ninhydrin sampling). Cleavage of the peptide-resin (222 mg, 32.4 μmol) gave, after purification by preparative reverse-phase HPLC (20−35% B over 60 min at 30 mL/min), 30.7 mg (8.1 μmol) of **3** (25% of theoretical yield), which was characterized by electrospray MS to give a mass of 3810 ± 1 Da [calcd 3807 Da (monoisotopic), 3810 Da (average isotope composition)].

Peptide Segments 2, 4, and 8. Peptide segments corresponding to the C-terminal regions of the target molecules were synthesized on MBHA resin to give peptide amides. The first residue coupled to the resin was Boc-Lys(Fmoc)-OH; all other lysine residues were coupled as Boc-Lys(2-Cl-Z)-OH. After completion of the stepwise chain assembly, the Fmoc protecting group was removed from the side chain amine of the carboxyl-terminal lysine by two 5 min treatments with 20% piperidine/DMF to yield **16**. [(*N*-(2-Cl-Z)-Amino)oxy]acetic acid (2.0 mmol) was dissolved in 4 mL of DMF and activated as the succinimide ester (**17**) by the addition of hydroxysuccinimide (2.0 mmol) and DIC (2.0 mmol). After an activation period of 1−2 h, without isolation of **17**, the mixture was added to the peptide-resin (**16**) and coupled for 1−2 h. Alternatively, the peptide-resin **16** was 4-oxopentanoylated. 4-Oxopentanoic acid (2.0 mmol) was activated as the symmetric anhydride by dissolving in CH_2Cl_2 (4 mL), to which was added DIC (1.0 mmol). After activation for 15 min to form the symmetric anhydride **18**, the mixture was added to the peptide-resin and coupled for 30−60 min. After removal of the amino-terminal Boc group with neat TFA (two 1 min treatments) and neutralization with 10% DIEA in DMF (two 1 min treatments), the product peptides **19** and **20** were bromoacetylated by the method of Robey[27] (analogous to that used for the 4-oxopentanoic acid). Bromoacetic acid (2.0 mmol) was dissolved in CH_2Cl_2 (2 mL) to which was added DIC (1 mmol). After activation for 15 min to form the symmetric anhydride **21**, the mixture was diluted with DMF (2 mL), added to the peptide-resin, and coupled for 30 min. The product peptides **2**, **4**, and **8** (Scheme 4) were each deprotected and concomitantly cleaved from the resin by treatment with HF containing 4−5% *p*-cresol for 1 h at 0 °C. After removal of the HF under reduced pressure, the crude peptides were each precipitated in anhydrous Et_2O, dissolved in 50% aqueous AcOH, diluted with water, and purified immediately or after prior lyophilization.

$BrCH_2CO$-cMyc(386−437)-Lys-$^{\epsilon}$N($COCH_2ONH_2$) (2). The C-terminal cMyc segment, corresponding to residues 386−437 of the complete human cMyc protein[32] (NNEKAPKVVILKKATAYILSVQAEEQKLISEEDLLRKRREQLKQKLEQLRNS), was synthesized on a 0.30 mmol scale to give 1.69 g of unmodified peptide-resin (72% of theoretical yield after ninhydrin sampling). Cleavage of bromoacetylated and (aminooxy)acetylated peptide-resin (279 mg, 27.3 μmol) gave, after purification by preparative reverse-phase HPLC (32−47% B over 60 min at 30 mL/min), 17.0 mg (2.6 μmol) of **2** (10% of theoretical yield), which was characterized by electrospray MS to give a mass of 6,440 ± 1 Da [calcd 6436 Da (monoisotopic), 6441 Da (average isotope composition)].

$BrCH_2CO$-Max(55−106)-Lys-$^{\epsilon}$N($COCH_2CH_2COCH_3$) (4). The C-terminal Max segment, corresponding to residues 55−106 of the complete murine Max protein[33] (GEKASRAQILDKATEYIQYMRRKNDTLQQDIDDLKRQNALLEQQVRALEKAR), was synthesized on a 0.30 mmol scale to give 1.81 g of unmodified peptide-resin (80% of theoretical yield after ninhydrin sampling). Cleavage of bromoacetylated and 4-oxopentanoylated peptide-resin (287 mg, 29.3 μmol) gave, after purification by preparative reverse-phase HPLC (32−47% B over 60 min at 30 mL/min), 26.0 mg (4.0 μmol) of **4** (14% of theoretical yield) which was characterized by electrospray MS to give a mass of 6507 ± 1 Da [calcd 6503 Da (monoisotopic), 6508 Da (average isotope composition)].

$BrCH_2CO$-Max(55−106)-Lys-$^{\epsilon}$N($COCH_2ONH_2$) (8). The C-terminal lysine side chain unprotected peptide-resin **16** used for the synthesis of **8** was the same as that used for **4**. Cleavage of bromoacetylated and (aminooxy)acetylated peptide-resin (288 mg, 29.1 μmol) gave, after purification by preparative reverse-phase HPLC (32−47% B over 60 min at 30 mL/min), 17.4 mg (2.7 μmol) of **8** (9% of theoretical yield), which was characterized by electrospray MS to give a mass of 6481 ± 1 Da [calcd 6478 Da (monoisotopic), 6483 Da (average isotope composition)].

Chemical Ligation Reactions. All ligations were performed in 8 M urea and 0.1 M NaH_2PO_4 (pH 4.7) (adjusted with AcOH) and were monitored by reverse-phase analytical HPLC and electrospray MS.

Monomer cMyc(353−437) -Lys-$^{\epsilon}$N($COCH_2ONH_2$) (5). Reaction was initiated by combining **1** (10.16 mg, 2.48 μmol) and **2** (10.53 mg, 1.64 μmol) in 1.0 mL of the ligation buffer. The reaction was stirred at 4 °C for 24 h. The ligation product was purified by semipreparative HPLC (32−47% B over 60 min at 3 mL/min) to give 8.3 mg (0.80 μmol) of **5** as a white solid after lyophilization (49% theoretical yield, based on **2**). The final product was characterized by electrospray MS to give a mass of 10 396 ± 2 Da [calcd 10 390 Da (monoisotopic), 10 396 Da (average isotope composition)].

Monomer Max(22−106)-Lys-$^{\epsilon}$N($COCH_2CH_2COCH_3$) (6). Reaction was initiated by combining **3** (11.83 mg, 3.10 μmol) and **4** (12.44 mg, 1.91 μmol) in 1.0 mL of the ligation buffer. The reaction was stirred at 4 °C for 24 h. The ligation product was purified by semipreparative HPLC (32−47% B over 60 min at 3 mL/min) to give 9.5 mg (0.93 μmol) of **6** as a white solid after lyophilization (49% theoretical yield, based on **4**). The final product was characterized by electrospray MS to give a mass of 10 233 ± 2 Da [calcd 10 229 Da (monoisotopic), 10 236 Da (average isotope composition)].

Covalent Heterodimer cMyc−Max (7). Reaction was initiated by combining **5** (8.33 mg, 0.80 μmol) and **6** (9.70 mg, 0.95 μmol) in 800 μL of the ligation buffer. The reaction was stirred at 25 °C for 48 h. The ligation product was purified by semipreparative HPLC (32−47% B over 60 min at 3 mL/min) to give 3.4 mg (0.16 μmol) of **7** as a white solid after lyophilization (21% theoretical yield, based on **5**). The final product was characterized by electrospray MS to give a mass of 20 610 ± 3 Da [calcd 20 601 Da (monoisotopic), 20 614 Da (average isotope composition)].

Covalent Homodimer Max−Max (9). The three-component ligation was initiated by combining **3** (12.27 mg, 3.22 μmol), **4** (4.93 mg, 0.76 μmol), and **8** (5.94 mg, 0.92 μmol) in 600 μL of the ligation buffer. The reaction was stirred at 25 °C for 48 h. The ligation product was purified by semipreparative HPLC (32−47% B over 60 min at 3 mL/min) to give 3.1 mg (0.15 μmol) of **9** as a white solid after lyophilization (20% of theoretical yield, based on **4**). The final product was characterized by electrospray MS to give a mass of 20 425 ± 3 Da [calcd 20 416 Da (monoisotopic), 20 428 Da (average isotope composition)].

[4-(α-Mercaptobenzyl)phenoxy]acetic Acid, Dicyclohexylamine (DCHA) Salt (11). [4-(α-Mercaptobenzyl)phenoxy]acetic acid was synthesized by a procedure based on the method of Koenig.[34] A mixture of [4-(α-chlorobenzyl)phenoxy]acetic acid (**10**)[25] (7.5 g, 27 mmol), thiourea (2.3 g, 30 mmol), and ethanol (100 mL) was heated to reflux. After 4 h, 10 N NaOH (30 mL) was added and the reflux continued for 2−3 h. After cooling to room temperature, the reaction mixture was concentrated *in vacuo* to approximately one-half the original volume, acidified with concentrated HCl (to pH 2.0), and extracted with EtOAc (4 × 30 mL). The combined EtOAc extracts

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(33) Prendergast, G. C.; Lawe, D.; Ziff, E. B. *Cell* **1991**, *65*, 395−407.

(34) Koenig, N. H.; Sasin, G. S.; Swern, D. *J. Org. Chem.* **1958**, *23*, 1525−1530.

were washed with saturated NaCl (1 × 30 mL) and dried over $MgSO_4$. The volatile materials were removed *in vacuo*. The resulting oil was dissolved in EtOAc (100 mL) and any insoluble material filtered. DCHA (6.0 mL, 30 mmol) was added to the filtrate with stirring. Within a few minutes, a white solid began to precipitate. Et_2O (150 mL) was added and the suspension cooled at −20 °C for several hours. The resulting white solid was filtered, washed with Et_2O, and dried under vacuum to give 10.3 g (23 mmol) of **11** (84%): ^{1}H NMR ($CDCl_3$) δ 7.30 (m, 7H), 6.82 (d, 2H, J = 8.7 Hz), 5.39 (br s, 1H), 4.40 (s, 2H), 2.81 (m, 2H), 2.23 (br s, 1H, ex D_2O), 1.88−1.02 (comp m, 20H); FAB MS (cesium ion) calcd for [$C_{27}H_{37}NO_3S$, H^+] 456.2572, found 456.2572. Anal. Calcd for $C_{27}H_{37}NO_3S$: C, 71.17; H, 8.18; N, 3.07; S, 7.04. Found: C, 71.11; H, 8.41; N, 3.08; S, 7.09.

[4-[α-(N-*t*-Boc-Leu-S)benzyl]phenoxy]acetic Acid, DCHA Salt (12). [4-[α-(*N*-*t*-Boc-Leu-S)benzyl]phenoxy]acetic acid was synthesized by an adaptation of the procedure of Hojo and Aimoto.[35] A mixture of **11** (1.672 g, 3.67 mmol), *N*-*t*-Boc-leucine, succinimide ester (1.208 g, 3.68 mmol), DIEA (1.0 mL, 5.74 mmol), DMF (35 mL), and CH_2Cl_2 (4 mL) was stirred at room temperature for 24 h. The reaction mixture was poured into 1 N HCl (150 mL) and extracted with EtOAc (4 × 35 mL). The combined EtOAc extracts were washed with 1 N HCl (2 × 30 mL), H_2O (1 × 30 mL), and saturated NaCl (1 × 30 mL) and dried over $MgSO_4$. Volatiles were removed *in vacuo*. The resulting oil was purified by flash chromatography (925:50:25 $CHCl_3$:MeOH:AcOH) to give an oil contaminated with AcOH. To remove residual AcOH, the oil was dissolved in $CHCl_3$ (40 mL), washed with 0.1 N HCl (7 × 10 mL) and saturated NaCl (1 × 10 mL), and dried over $MgSO_4$. Volatiles were removed *in vacuo* to give 1.534 g (2.74 mmol) of [4-[α-(*N*-*t*-Boc-Leu-S)benzyl]phenoxy]acetic acid as an oil. This oil was dissolved in Et_2O (10 mL), to which was added DCHA (550 μL, 2.76 mmol). Hexane (100 mL) was added with stirring to separate **12** as a thick oil from any unreacted DCHA. Solvents were decanted from the oil, and the oil was dissolved in CH_2Cl_2 (30−40 mL). The resulting solution was concentrated *in vacuo* to give 1.593 g (2.38 mmol) of the salt **12** (65%) as a white foamy solid: ^{1}H NMR ($CDCl_3$) δ 7.22 (m, 7H), 6.81 (d, 2H, J = 7.5 Hz), 5.80 (s, 1H), 4.78 (d, 1H, J = 8.1 Hz, ex D_2O), 4.39 (m, 3H), 2.82 (m, 2H), 1.88−1.06 (comp m, 32H), 0.90 (d, 6H, J = 6.0 Hz); FAB MS (cesium ion) calcd for [$C_{38}H_{56}N_2O_6S$, H^+] 669.3937, found 669.3960. Anal. Calcd for $C_{38}H_{56}N_2O_6S$: C, 68.23; H, 8.44; N, 4.19; S, 4.79. Found: C, 68.09; H, 8.59; N, 3.82; S, 4.91.

[(*N*-(2-Cl-Z)-Amino)oxy]acetic Acid. (Aminooxy)acetic acid hemihydrochloride (2.80 g, 25.6 mmol) was gradually added to 5% aqueous $NaHCO_3$ (200 mL). *N*-[(2-Chlorobenzyloxycarbonyl)oxy]succinimide (8.00 g, 28.2 mmol) was dissolved in tetrahydrofuran (100 mL) and added to the aqueous (aminooxy)acetic acid/$NaHCO_3$ solution. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*. The resulting aqueous solution was adjusted to a pH of 2.0 with 5 M HCl and extracted with EtOAc (4 × 50 mL). The combined EtOAc extracts were washed with saturated NaCl and dried over $MgSO_4$. The volatiles were removed *in vacuo* to give a thick oil which was crystallized from $CHCl_3$/petroleum ether to give 6.1 g (92%) of [(*N*-(2-Cl-Z)-amino)oxy]acetic acid as a white solid: ^{1}H NMR ($CDCl_3$) δ 7.50 (m, 2H), 7.39 (m, 2H), 5.18 (s, 2H), 4.32 (s, 2H). Anal. Calcd for $C_{10}H_{10}NO_5Cl$: C, 46.26; H, 3.88; N, 5.39. Found: C, 45.96; H, 3.96; N, 5.44.

Electrophoretic Mobility Gel Shift Assay. Bacterial Max b/HLH/Z was purified as described previously.[8] HPLC-purified covalent Max homodimer and covalent cMyc−Max heterodimer were dissolved in 150 mM KCl, 10% glycerol, and 5 mM $MgCl_2$. The probe and the specific competitor were a 20-base pair (bp) duplex DNA of the sequence ctactagCACGTGctagtag. Equimolar amounts of annealed [^{32}P]phosphorylated probe and protein were incubated at a final concentration of 0.5 μM with 10% glycerol, 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)−KOH (pH 5.5), 150 mM KCl, and 1 mM $MgCl_2$ for 1 h at room temperature before being resolved in a prerun 0.5x TBE 6% acrylamide 29:1 acrylamide:bis(acrylamide) gel at 15 V/cm for 3 h. Gels were visualized by autoradiography. The competitor DNA, where present, was in a 100-fold molar excess. The nonspecific competitor is a duplex DNA with the unrelated sequence gaaaatcac-ccaactgca.

Acknowledgment. This work was supported by funds from the NIH (S.B.H.K.). A.F.D. was supported as a David Rockefeller graduate student. We gratefully acknowledge the assistance of Dr. Gary Siuzdak and Dr. Raj K. Chandra, the Scripps Research Institute, for FAB MS measurements and for elemental analyses, respectively; Dr. Reyna Simon for critical comments on the manuscript; Dr. Mark Surles, Interactive Simulations, Inc. (creator of the molecular modeling program *Sculpt*) and Philip Dawson for creating Figure 2; and our colleagues at The Scripps Research Institute for numerous helpful discussions.

JA9435145

(35) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111−117.